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(1) I am fully conversant both with the Japanese and English languages.

(2) I have carefully compared the attached English language translation of Japanese Patent Application Number 2002-293512, filed October 7, 2002, with the original Japanese-language patent application.

(3) The translation is, to the best of my knowledge, and belief, an accurate translation from the original into the English language.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the matter with which this translation is used.

Date: February 13, 2006 Toshio Shimizu

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[Document name] Specification
[Title of the invention] Chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase gene
[Scope of claim for patent]

[Claim 1] A gene comprising either one of base sequences of:

(a) a base sequence described in SEQ.ID.NO: 1,

(b) a base sequence which encodes a protein having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the base sequence is a base sequence in which one or a plural number of base(s) is/are deleted, added or substituted from the base sequence described in SEQ.ID.NO: 1,

(c) a base sequence which encodes a protein having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity and hybridizes with the base sequence described in SEQ.ID.NO: 1 under stringent conditions,

(d) a base sequence which encodes an amino acid sequence described in SEQ.ID.NO: 2,

(e) a base sequence which encodes an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence in which one or a plural number of amino acid(s) is/are deleted, added or substituted from the amino acid sequence described in SEQ.ID.NO: 2, or

(f) a base sequence which encodes an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence having at least 60% or more of homology with the amino acid sequence described in SEQ.ID.NO: 2.

[Claim 2] A protein comprising either one of amino acid sequences of:

(a) an amino acid sequence described in SEQ.ID.NO: 2,

(b) an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence in which one or a plural number of amino acid(s) is/are deleted, added or substituted from the amino acid sequence described in

SEQ.ID.NO: 2, and
(c) an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence having at least 60% or more of homology with the amino acid sequence described in SEQ.ID.NO: 2.

[Claim 3] A signal peptide comprising an amino acid sequence described in SEQ.ID.NO: 7 for a resting strain reaction using a protein according to Claim 2.

[Claim 4] A DNA which encodes a signal peptide as defined in Claim 3, which comprises a base sequence described in SEQ.ID.NO: 8.

[Claim 5] A vector containing the gene according to Claim 1.

[Claim 6] The vector according to Claim 5, wherein said vector further contains the DNA as defined in Claim 4.

[Claim 7] The vector according to Claim 6, wherein said vector is plasmid pKK-EnHCH1.

[Claim 8] A transformant containing the gene as defined in Claim 1 or the vector as defined in any one of Claims 5 to 7.

[Claim 9] The transformant according to Claim 8, wherein a host is *E. coli*.

[Claim 10] The transformant according to Claim 9, wherein the host is *E. coli* JM109 strain or DH5α strain.

[Claim 11] A process for the preparation of the protein as defined in Claim 2, which comprises using the gene as defined in Claim 1.

[Claim 12] A process for the preparation of an optically active compound which comprises using the protein as defined in Claim 2.

[Claim 13] A process for the preparation of an optically active compound which comprises using the transformant defined in any one of Claims 8 to 10.

[Detailed description of the invention]

[0001]

[Technical field to which the invention belongs]

The present invention relates to a hydroxy carboxylic ester asymmetric hydrolase (Hydroxy Carboxylic ester Hydrolase: hereinafter abbreviated to as "EnHCH"), a gene encoding the same, a recombinant vector containing the gene, a transformant transformed by the recombinant vector and a process for producing the enzyme using the transformant, which EnHCH is a biological catalyst useful for the preparation of an optically active chlorohydrin, optically active 3-hydroxy-γ-butyrolactone, optically active hydroxycarboxylic acid, and its antipode alkyl ester which is a useful chiral building block in the synthesis of an optically active compound to be used for medicines, agricultural chemicals, and strongly dielectic liquid crystal, etc. Moreover, it relates to a process for the preparation of an optically active material using the enzyme and the transformant.

[0002]

[Prior art]

As a process for preparing an optically active compound, it have been usually employed a process in which corresponding racemic mixture is treated by an optically resolving agent to resolve into an optically active material in addition to a chemical synthesis method in which the corresponding optically active starting compound is converted into the desired compound, but recently, it has been reported to produce the optically active compound by a biological optical resolution utilizing an asymmetric reduction or asymmetric hydrolysis of a racemic compound with a microorganism or an enzyme.

[0003]

As a method for preparing optically active 4-chloro-3-hydroxycarboxylic ester, E. Santaniello, et al. have reported a process for prepaing S-ethyl 4-chloro-3-hydroxybutanoate from ethyl 4-chloro-3-oxobutanoate by asymmetric reduction using baker's yeast (Non-Patent Literature 1). Also, Takahashi et al. have reported a process for the preparation of optically active ethyl 4-chloro-3-hydroxybutanoate from ethyl 4-chloro-3-oxobutanoate by asymmetric reduction using microorganisms (Patent Literature 1).

[0004]

As a preparation process using an enzyme, Peters et al. have reported a process for the preparation of S-methyl 3-hydroxybutanoate and R-ethyl 4-chloro-3-hydroxybutanoate from methyl 3-oxobutanoate or ethyl 4-chloro-3-oxobutanoate by asymmetric reduction using carbonyl reductase for *Rhodococcus erythropolis* (Non-Patent Literatures 2, 3). Moreover, Shimizu et al. have reported a preparation process of R-ethyl 4-chloro-3-hydroxybutanoate by asymmetric reduction using aldehyde reductase for *Sporoboromyces salmonicolor* AKU4429 strain (Non-Patent Literatures 4, 5).

[0005]

However, in the preparation method of an optically active β-hydroxy ester compound from a prochiral β-keto ester compound by asymmetric reduction using these microorganisms or enzymes, an expensive coenzyme such as NADH (nicotinamide adenine dinucleotide) or NADPH (nicotinamide adenine dinucleotide phosphate), etc. are required for the reaction, and its oxidized product is required to be converted again into a reduced material whereby an enzyme such as glucose oxidase or formic acid dehydrogenase, etc. is separately required. Moreover, the above reaction step becomes a rate-determining reaction, and thus, the above-mentioned process cannot be said to be an industrially useful process.

[0006]

As a process for the preparation of optically active 3-hydroxybutanoate, it has been known a process for the preparation of S-3-hydroxybutanoate from acetoacetate by asymmetric reduction using microorganisms such as yeast (Non-Patent Literatures 6) or *Halobacterium halobium* (Non-Patent Literatures 7). However, in this method, an expensive coenzyme such as NADH (nicotinamide adenine dinucleotide) or NADPH (nicotinamide adenine dinucleotide phosphate), etc. is required for the reaction.

[0007]

When the above-mentioned *Halobacterium halobium* is acted on racemic 3-hydroxybutanoate, it has been reported that R-3-

hydroxybutanoate remains by carboxylate hydrolysis reaction. It has been not known, however, a process for the preparation of S-3-hydroxybutanoate with high optical purity from racemic 3-hydroxybutanoate using a microorganism having a stereospecific ester resolution activity.

[0008]

As a process for the preparation of optically active 2-hydroxybutanoate, it has been known a process from 2-hydroxyhexadecanoic ester or 2-hydroxytetracosanoic ester by an interesterification reaction of a secondary alcohol using lipase (Non-Patent Literature 8). However, it has been not known a process for the preparation of optically active 2-hydroxybutanoate by hydrolysis reaction of a carboxylate.

[0009]

Also, as a process for the preparation of optically active lactic acid utilizing microorganisms or enzymes, there have been known a fermentation method from glucose using lactic acid bacterium (Non-Patent Literatures 9, 10) or a preparation process from 2-halopropionic acid by a dehalogenation enzyme using a Pseudomonas genus microorganism (Non-Patent Literature 11). However, it has been not known a process for the preparation of optically active lactate or lactic acid by stereospecifically hydrolyzing a carboxylate.

[0010]

Also, as a process for the preparation of optically active tetrahydrofuran-2-carboxylate by utilizing an enzyme, it has been known a process for the preparation of the same from a racemic mixture by stereospecific hydrolysis using various kinds of protease, lipase (Patent Literatures 2, 3), esterase (Patent Literature 4) each derived from nature. However, there is no process which can obtain R-methyl tetrahydrofuran-2-carboxylate with high optical purity. Also, the above process requires an expensive enzyme.

[0011]

By the reasons as stated above, it has been strongly desired to culture a separated strain, to produce an enzyme with high properties with an inexpensive cost and a large amount whereby applying the enzyme to a process for the preparation of optically active hydroxycarboxylic acid and its antipode alkyl ester to make the process simple and ease.

[0012]

Also, if a gene of the enzyme can be subjected to cloning, it is possible to produce the enzyme with an inexpensive cost and a large amount by using a genetic engineering technique, so that is has been strongly desired to subject the gene encoding the enzyme to cloning.

[0013]

As a process for solving the above problems, the present inventors have already found *Enterobacter* sp. DS-S-75 strain (FERM BP-5494) separated from soil which is a bacterium belonging to the genus *Enterobacter* and having an activity of subjecting to stereoselective ester hydrolysis reaction and proposed a process for converting S-4-chloro-3-hydroxybutanoate into optically active 3-hydroxy-γ-butyrolactone by acting microorganisms or a product thereof on a chlorohydrin compound such as racemic 4-chloro-3-hydroxybutanoate to effect stereoselective dechlorination and ester hydrolysis reaction, and to recover a remaining another R-4-chloro-3-hydroxybutanoate simultaneously (see Patent Literatures 5 and 6, Non-Patent Literature 12).

[0014]

They have further studied about a substrate specificity in the above-mentioned microorganism raction, and as a result, they have found that the microorganisms have characteristics of stereoselectively degrading various kinds of hydroxycarboxylic esters (Patent Literature 7). They have further studied, and as a result, they have succeeded in purifying asymmetric hydrolase (EnHCH) which participates in the above-mentioned microorganism reaction from the cells of the microorganism and in obtaining a EnHCH gene encoding the same.

[0015]

[Patent Literature 1]

Japanese Laid-Open Patent Application No. Sho. 61-146191

[Patent Literature 2]

WO 01/92553-A pamphlet

[Patent Literature 3]

WO 01/92554-A pamphlet

[Patent Literature 4]

Japanese Laid-Open Patent Application No. 2002-171994

[Patent Literature 5]

Japanese Laid-Open Patent Application No. Hei. 9-47296

[Patent Literature 6]

U.S. Patent No. 5,776,766 specification

[Patent Literature 7]

Japanese Patent Application No. Hei. 13-391726 specification

[Non-Patent Literature 1]

Written by E. Santaniello et al., Journal of Chemical Research (J. Chem. Research), 1984, pp. 132-133

[Non-Patent Literature 2]

Written by J.Peters et al., Applied Microbiology Biotechnology (Appl. Microbiol. Biotechnol.), 1992, vol. 38, pp. 334-340

[Non-Patent Literature 3]

Written by T.Zelinski et al., Journal of Biotechnology (J. Biotechnol.), 1994, vol. 33, pp. 283-292

[Non-Patent Literature 4]

Written by Shimizu et al., Biotechnology Letter (Biotechnol. Lett.), 1990, vol. 12, pp. 593-596

[Non-Patent Literature 5]

Written by Shimizu et al., Applied Microbiology Biotechnology (Appl. Microbiol. Biotechnol.), 1990, vol. 56, pp. 2374-2377

[Non-Patent Literature 6]

Written by Hamdani et al., Tetrahedron: Asymmetery, 1991, vol. 2, pp. 867-870

[Non-Patent Literature 7]

Written by Ehrler and Seebach, Helvetica Chimica Acta (Helv. Chim. Acta), 1989, vol. 72, pp. 793-799

[Non-Patent Literature 8]

Written by Sugai et al., Yukigosei Kagakukaishi (Journal of Organic Syntesis Chemistry Association), 1995, vol. 53, pp. 48-58

[Non-Patent Literature 9]

Written by Brin, Biochemical Preparation (Biochem. Prepn.), 1953, vol. 3, p. 61;

[Non-Patent Literature 10]

Written by Andersen and Greaves, Industrial Engineering Chemistry (Ind. Eng. Chem.), 1942, vol. 34, p. 34

[Non-Patent Literature 11]

Written by Soda et al., Biodegradation, 1995, vol. 6, pp. 223-227

[Non-Patent Literature 12]

Written by Suzuki et al., Enzyme and Microbial Technology, 1999, vol. 24, pp. 13-20

[0016]

[Problems to be solved by the invention]

The present invention is to provide a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase (EnHCH) and a gene thereof in which stereoselectivity is improved, which can markedly improve catalytic activity of a microorganism as compared with the conventional one, and can produce an optically active compound with an industrial scale by using microorganisms prepared by genetic engineering. The present invention is also to provide a vector including the gene, a transformant, and a process for producing an optically active isomer using the enzyme. The present invention is further to provide a gene which encodes a signal peptide of EnHCH which stably express gene in the transformant.

[0017]

[Means to solve the problems]

According to the present invention, it is provided a gene comprising either one of base sequences of:

(a) a base sequence described in SEQ.ID.NO: 1,

(b) a base sequence which encodes a protein having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the base sequence is a base sequence in which one or a plural number of base(s) is/are deleted, added or

substituted from the base sequence described in SEQ.ID.NO: 1,
(c) a base sequence which encodes a protein having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity and hybridizes with the base sequence described in SEQ.ID.NO: 1 under stringent conditions,
(d) a base sequence which encodes an amino acid sequence described in SEQ.ID.NO: 2,
(e) a base sequence which encodes an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence in which one or a plural number of amino acid(s) is/are deleted, added or substituted from the amino acid sequence described in SEQ.ID.NO: 2, and
(f) a base sequence which encodes an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence having at least 60% or more of homology with the amino acid sequence described in SEQ.ID.NO: 2.

Also, according to the present invention, it is provided a protein comprising either one of amino acid sequences of:
(a) an amino acid sequence described in SEQ.ID.NO: 2,
(b) an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence in which one or a plural number of amino acid(s) is/are deleted, added or substituted from the amino acid sequence described in SEQ.ID.NO: 2, and
(c) an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence having at least 60% or more of homology with the amino acid sequence described in SEQ.ID.NO: 2.

Also, according to the present invention, it is provided a signal peptide comprising an amino acid sequence described in SEQ.ID.NO: 7 for a resting strain reaction using a transformant of the present invention.

Also, according to the present invention, it is provided a DNA which encodes a signal peptide comprising a base sequence described in SEQ.ID.NO: 8.

Also, according to the present invention, it is provided a vector containing the gene of the present invention. The vector preferably contains the DNA which encodes a signal peptide, and is further preferably plasmid pKK-EnHCH1.

Also, according to the present invention, it is provided a transformant containing the above-mentioned vector. The host is preferably *E. coli*, more preferably *E. coli* JM109 strain or DH5α strain.

Also, according to the present invention, it is provided a process for the preparation of the protein of the present invention which comprises using the gene or protein of the present invention.

Also, according to the present invention, it is further provided a process for the preparation of an optically active compound using the protein or transformant of the present invention.

[0018]

[Embodiments of the invention]

In the following, embodiments of the present invention are explained in more detail.

The present invention relates to a gene comprising either one of base sequences of:

(a) a base sequence described in SEQ.ID.NO: 1,

(b) a base sequence which encodes a protein having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the base sequence is a base sequence in which one or a plural number of base(s) is/are deleted, added or substituted from the base sequence described in SEQ.ID.NO: 1,

(c) a base sequence which encodes a protein having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity and hybridizes with the base sequence described in SEQ.ID.NO: 1 under stringent conditions,

(d) a base sequence which encodes an amino acid sequence described in SEQ.ID.NO: 2,

(e) a base sequence which encodes an amino acid sequence

having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence in which one or a plural number of amino acid(s) is/are deleted, added or substituted from the amino acid sequence described in SEQ.ID.NO: 2, and
(f) a base sequence which encodes an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence having at least 60% or more of homology with the amino acid sequence described in SEQ.ID.NO: 2.

[0019]

In the present specification, the terms "one or a plural number of amino acid(s) is/are deleted, added or substituted" mean that, for example, an optional number of 1 to 20 amino acids, preferably 1 to 15 amino acids, more preferably 1 to 10 amino acids, further preferably 1 to 5 amino acids is/are deleted, added or substituted. In the present specification, the terms "one or a plural number of base(s) is/are deleted, added or substituted" mean that, for example, an optional number of 1 to 20 bases, preferably 1 to 15 bases, more preferably 1 to 10 bases, further preferably 1 to 5 bases is/are deleted, added or substituted.

[0020]

In the present specification, the terms "which can hybridize under stringent conditions" mean a nucleic acid obtained by using a colony hybridization method, a plaque hybridization method, or a southern blot hybridization method, etc. which use a nucleic acid such as DNA or RNA as a probe. More specifically, there may be mentioned a DNA which is identifiable by subjecting to hybridization using a DNA derived from a colony or plaque, or a filter to which a fragment of the DNA is fixed, in the presence of 0.7 to 1.0M NaCl at 65°C, and then, washing the filter with 0.1 to 2-fold of a SSC solution (a composition of the SSC solution with a 1-fold concentration comprises 150 mM of sodium chloride and 15 mM of sodium citrate) at 65°C. The hybridization can be carried out according to the method as described in Molecular

Cloning: A Laboratory Mannual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., 1989. Hereinafter abbreviated to as "Molecular Cloning 2nd Ed.") and the like.

[0021]

As the DNA which can hybridize under stringent conditions, there may be mentioned a DNA having homology with a certain degree or more with a base sequence of the DNA to be used as a probe, and the homology is, for example, 60% or more, preferably 70% or more, more preferably 80% or more, further preferably 90% or more, particularly preferably 95% or more, most preferably 98% or more.

[0022]

In the present specification, the terms "chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase" mean a protein having amino acid sequence of SEQ.ID.NO: 2 with a molecular weight of 75.0 kDa, which is a homodimer having a molecular weight of 37.5 kDa, with an isoelectric point of 6.7.

[0023]

In the present specification, the terms "chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity" mean an activity which is to subject to stereoselectively dechlorination and hydrolysis of a racemic chlorohydrin represented by the following formula [1]:

[0024]

[Formula 1]

$$Cl-CH_2-CHOH-CH_2-COOR^1 \qquad [1]$$

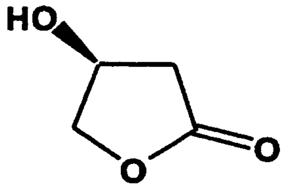
[0025]

(wherein R^1 represents a C_1 to C_4 alkyl group.)

to form S-3-hydroxy-γ-butyrolactone represented by the formula [2]:

[0026]

[Formula 2]



[2]

and to remain R-chlorohydrin; to subject to hydrolysis stereoselectively of a racemic hydroxycarboxylic ester represented by the following formula [3]:

[0027]

[Formula 3]

$$R^1\text{-}(CH_2)_n\text{-}CHOH\text{-}(CH_2)_m\text{-}COOR^2$$

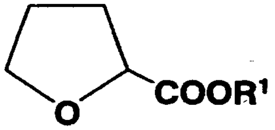
[3]

[0028]

(wherein R^1 represents a methyl group or a benzyl group; R^2 represents a C1 to C4 alkyl group; when m is 0 and n is 0 or 1, or m is 1 and n is 0.)
to form an optically active hydroxycarboxylic acid and to remain its antipode ester; or to subject to hydrolysis stereoselectively of a racemic tetrahydrofuran-2-carboxylic ester represented by the following formula [4]:

[0029]

[Formula 4]



[4]

[0030]

(wherein R^1 represents a C1 to C4 alkyl group.)
to remain R-tetrahydrofuran-2-carboxylic ester.

[0031]

A method of obtaining a gene of the present invention is not specifically limited. An objective gene can be isolated by preparing a suitable probe or primer based on information of the base sequence of SEQ.ID.NO: 1 or the amino acid sequence of SEQ.ID.NO: 2 disclosed in the present specification, and subjecting to screening of a DNA library in which said gene is expected to be present by using the above probe or primer.

[0032]

More specifically, the EnHCH gene shown in SEQ.ID.NO: 1 of the present invention can be separated from a chromosome DNA of a microorganism DS-S-75 strain (FERM BP-5494) belonging to the genus *Enterobacter*. The DNA fragment having the EnHCH gene can be obtained from the gene donor, for example, based on the partial amino acid sequence of a polypeptide chain of the purified enzyme EnHCH. That is, the purified enzyme is digested by endopeptidase, a part of the amino acid sequence of the respective fagments is determined by a protein sequencer, and a primer for the polymerase chain reaction (PCR) is synthesized based thereon. Then, PCR is carried out using a chromosome DNA of a DS-S-75 strain belonging to the genus Enterobacter as a template to amplify a part of the EnHCH gene whereby the base sequence is clarified. By using the obtained partial base sequence of the EnHCH gene as a probe, a DNA fragment of the objective gene can be obtained by using the hybridization method from the DNA library prepared from the chromosome DNA of the gene donor according to the conventional manner.

[0033]

As a method for determining the DNA base sequence of the obtained EnHCH gene, a dideoxy sequence method may be mentioned. This method includes various kinds of methods generally used in the field of genetic engineering such as a method of amplifying a gene by PCR or a method of deleting a nucleic acid(s) by a nuclease, or the like. According to these methods, an open reading flame (ORF) which encodes whole amino acids in the objective DNA base sequence shown by SEQ.ID.NO: 1 can be confirmed.

[0034]

The DNA which encodes EnHCH of the present invention is characterized in that an amino acid sequence having an EnHCH activity substantially contains a base sequence which encodes the polypeptide shown in SEQ.ID.NO: 2. Here, as long as it has an EnHCH activity, one or several number of amino acid(s) may be deleted, added or substituted in the amino acid sequence shown in SEQ.ID.NO: 2. For example, modification of DNA can be optionally carried out by the method known in the art such as a site-specific mutation introducing method using a synthetic oligonucleotide, so that some of the amino acids constituting the amino acid sequence which encodes the DNA are deleted, added or substituted. Also, by using the DNA shown in SEQ.ID.NO: 1 or a DNA which is prepared by optionally modifying the above DNA as a template, a DNA in which mutation is introduced at random can be obtained by effecting a PCR method in the presence of Mn^{2+} ion (generally a concentration of 0.5 to 10 mM), or making a concentration of a specific nucleotide low. Of these DNAs thus obtained, it is needless to say that a material which encodes a protein having an EnHCH activity is included in the present invention.

[0035]

Also, the present invention relates to a protein comprising either one of amino acid sequences of:

(a) an amino acid sequence described in SEQ.ID.NO: 2,

(b) an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence in which one or a plural number of amino acid(s) is/are deleted, added or substituted from the amino acid sequence described in SEQ.ID.NO: 2, and

(c) an amino acid sequence having a chlorohydrin and hydroxycarboxylic ester asymmetric hydrolase activity where the amino acid sequence is an amino acid sequence having at least 60% or more of homology with the amino acid sequence described in SEQ.ID.NO: 2.

[0036]

In the present specification, "an amino acid sequence having at least 60% or more of homology with the amino acid sequence described in SEQ.ID.NO: 2" is not specifically limited so long as it has homology with the amino acid sequence of SEQ.ID.NO: 2 of 60% or more, for example, it means 60% or more, preferably 70% or more, more preferably 80% or more, further preferably 90% or more, particularly preferably 95% or more, most preferably 98% or more.

[0037]

A purification method of the enzyme according to the present invention is not specifically limited, and purification can be carried out by optionally combining purification methods generally employed in the art. For example, after crushing cells of the microorganisms by ultrasonic wave, salt out by ammonium sulfate is carried out, and a dissolved material of the precipitate is purified by optionally combining hydrophobic chromatography, anion exchange chromatography and gel filtration chromatography, until it becomes single by sodium dodecylsulfate-polyacrylamide gel electrophoresis (hereinafter abbreviated to as "SDS-PAGE").

[0038]

The present invention also relates to a signal peptide comprising 25 amino acids shown in SEQ.ID.NO: 7 which is located at upstream of the EnHCH and a gene encoding the same (shown by SEQ.ID.NO: 8). According to this signal peptide, a transformant of *E. coli* into which the above gene is introduced having a high EnHCH activity can be obtained in the state of a cell culture broth. When the cell is removed, the activity is lost. It means that no enzyme is present in the medium, so that it is easy to prevent from excessive reaction or to carry out extraction of the product. Also, when necessity arises, only the gene of said signal sequence is connected to the other useful gene.

[0039]

The present invention is further to provide an expression plasmid containing a base sequence of a gene which encodes an amino acid sequence showing an EnHCH activity. By incorporating a necessary portion of a DNA fragment which carries gene information of EnHCH into a vector, and it is incorporated into a host cell, a transformant can be obtained. As such a vector, preferred is a material to which a suitable selective marker, etc. which is capable of being self-replication in a host cell and is capable of selecting a recombinant host cell alone is provided, and the gene of the present invention can be expressed in a suitable host cell. Moreover, such a vector may be those which can be easily prepared from a conventionally known vector by a person skilled in the art using a conventionally known technique, or may be those which are commercially available. Particularly preferred is plasmid pKK-223-3.

[0040]

The present invention is further to provide a microorganism having an ability of producing an EnHCH activity, which is transformed by a DNA which encodes the polypeptide an amino acid sequence of which is substantially shown in SEQ.ID.NO: 2.

[0041]

As a host cell to be used, any material can be used without restriction so long as it is transformed by the resulting recombinant vector and can express the gene of the present inventoin. Such a host cell may include gram negative bacteria as well as gram positive bacteria so long as it can accomplish expression of the gene of the present invention along with the object of the same, and further may include either of a procaryotic cell or eucaryotic cell, or either of cells derived from animals or cells derived from plants.

[0042]

As a host cell which can be used specifically in the present invention, there may be mentioned, for example, microorganisms selected from the group consisting of *E. coli* (*Escherichia coli*), the genuses *Enterobacter*, *Saccharomyces*, *Xanthomonas*, *Acetobacter*, *Pseudomonas*, *Gluconobacter*, *Azotobacter*, *Rhizobium*, *Klebsiella*, *Salmonella* and *Serratia*, and preferably *E. coli* is used. Particularly preferred is *E. coli* DH5α. DH5α (pKK-EnHCH1) is deposited to International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology as a deposition number FERM BP-08466.

[0043]

The transformant can be obtained with a large scale by carrying out cultivation in a suitable culture medium. The culture medium may be any of usual media which contain a carbon source, a nitrogen source, inorganic substances, etc., necessary for growth of microorganisms. For example, there may be used as a carbon source, hydrocarbon such as glucose, fructose, etc., an alcohol such as glycerol, mannitol, sorbitol, propylene glycol, etc., an organic acid such as acetic acid, citric acid, malic acid, maleic acid, fumaric acid, gluconic acid and a salt thereof, or a mixture thereof. As a nitrogen source, there may be mentioned an inorganic nitrogen compound such as ammonium sulfate, ammonium nitrate, ammonium phosphate, etc., and an organic nitrogen compound such as urea, peptone, casein, yeast extract, meat extract, corn steep liquor, etc., and a mixture thereof. In addition, as an inorganic salt, phosphate, magnesium salt, potassium salt, manganese salt, iron salt, zinc salt, copper salt, etc. may be added and vitamins may be further added, if necessary. Also, as an enzyme derived additive to obtain a transformant cells having a high enzyme activity, antibiotics such as ampicillin, kanamycin, chloramphenicol, etc. may be added to a culture broth to express an objective DNA effectively depending on the strain to be used in a nutrient medium such as the above-mentioned medium, peptone medium, bouillon medium, etc., or an activation inducer of a promoter such as isopropyl β-D(-)-thiogalactopyranoside (IPTG), etc. may be used. Cultivation may be carried out under aerobic conditions by controlling an optional range at a pH of 4 to 10 and a temperature of 20 to 60°C for 1 to 5 days, and it is more effective to carry out cultivation under optimum conditions for the transformant to be used.

[0044]

Extraction of the enzyme from the resulting cells of the EnHCH-producing transformant can be carried out by the following method. According to the methods of 1) a mechanical (physical) method by French press or ultrasonic wave pulverization, 2) an enzyme treatment method such as lysozyme, 3) an autodissolution method, 4) an extraction method utilizing an osmotic pressure, etc., the transformant is broken. Also, *E.*

coli DH5α (pKK-EnHCH1) can be utilized as a high activity enzyme in the state of a culture broth without pulverizing the cells.

[0045]

The enzyme produced by the present process is not limited only to those which are accorded with the polypeptide sequence of EnHCH in the origin of the genus *Enterobacter*, and it may include any polypeptides obtained by gene recombinant methods such as base sequence transformation or gene transformation, and show an EnHCH activity. That is, those in which one or several number of amino acids in the peptide sequence is/are deleted, or one or several number of amino acids in the peptide sequence is/are substituted by other amino acid(s).

[0046]

The thus produced transformant which produces a stereo-selective hydrolase with a large amount can be prepared with an industrial scale in an inexpensive medium, and it can be utilized as a bioreactor for the preparation of R-4-chloro-3-hydroxycarboxylic ester, S-3-hydroxy-γ-butyrolactone, optically active hydroxycarboxylic acid, and its antipode ester, R-tetrahydrofuran-2-carboxylic ester, etc.

[0047]

In particular, *E. coli* DH5α (pKK-EnHCH1) shows higher reactivity as compared with a gene donor (DS-S-75 strain of the genus *Enterobacter*), and it is particularly possible to obtain S-3-hydroxy-γ-butyrolactone, R-3-hydroxybutyric acid, and S-2-hydroxybutyric acid within a short period of time with a high optical purity.

[0048]

[EXAMPLES]

In the following, the present invention is explained by referring to Examples in more detail. The present invention is not at all limited by these Examples.

[0049]

[Example 1] Purification of EnHCH enzyme

Measurement of an enzyme activity was carried out by using 1% (v/v) methyl 4-chloro-3-hydroxybutanoate as a substrate and reacting in 0.5 M potassium phosphate buffer (pH 7.15) at 30°C, and liberated chloro ions were colorimetrically determined according to the method of Iwasaki et al. (Bull. Chem. Soc. Japan, 25, 226 (1952)). An enzyme amount which liberates 1 μmol of chloro ion within one minute is defined to be 1U. Determination of the protein was carried out by measuring an absorption at 280 nm.

[0050]

DS-S-75 strain (FERM BP-5494) belonging to the genus *Enterobacter* was cultured in a PYG medium (1% peptone, 1% yeast extract and 1% glycerol, pH 7.2), and cells were prepared by centrifugation.

[0051]

The resulting wet cells were pulverized by an ultra high-pressure cell pulverizing device, and then, cell residue were removed by centrifugation to obtain cell-less extract. To the extract was added ammonium sulfate, and precipitated fraction at 50% saturation was recovered. Moreover, it was applied to hydrophobic chromatography by Butyl-toyoperl equilibrated with 10 mM tris-sulfate buffer (pH 7.8) containing 1.6 M of ammonium sulfate. A concentration of ammonium sulfate was lowered to 0 M, and an active fraction of the enzyme was recovered. To the fraction was added ammonium sulfate, and precipitates at 80% saturation were dissolved in 10 mM tris-sulfate buffer (pH 7.8), and dialyzed by the same buffer. After dialysis, the resulting material was applied to ion exchange column chromatography by DEAE-sepharose equilibrated with the same buffer. Dissolution was carried out with concentration gradient (10-200 mM) of the same buffer, and active fractions of the enzyme were recovered. These fractions were subjected to desalting concentration by using ultra-filtratoin membrane (Macrosep 10K, available from Nihon Pall Ltd.). The concentrate was applied to gel filtration column chromatography using Sephadex-G150 equibrated with 0.1 M potassium phosphate buffer, to recover active fractions of the enzyme.

[0052]

The resulting asymmetric hydrolase standard product was analyzed by SDS-PAGE, and as a result, it became a single band and the molecular weight was 37.5 kDa. As results of gel filtration column chromatography and Native-PAGE using a separation gel to which 10-20% of concentration gradient had been applied, the molecular weight was 75 kDa. From these results, it cound be found that the enzyme was homo dimer of subunits having a molecular weight of 37.5 kDa.

[0053]

Summary of the purification was shown in Table 1, specific activity of the purified enzyme was 7690 U/mg-protein, and was 221-fold as compared to the crude enzyme extract.

[0054]

[Table 1]

	Volume (ml)	Total protein weight (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purified degree (fold)
Crude extract	90	2540	88300	34.8	100	1.0
0-50% ammonium sulfate precipitate	200	1680	53800	32.0	61.0	0.90
Butyl toyopearl	173	142	31800	269	43.2	7.70
0-80% ammonium sulfate precipitate	38.3	132	23000	174	26.0	5.0
DEAE-sepharose	75.3	5.95	28200	4750	32.0	136
Sephadex G-150	16.8	2.54	19500	7690	22.1	221

[0055]

[Example 2] Analysis of partial amino acid sequence of EnHCHenzyme

Purified EnHCH enzyme in an amount of 45 μg was treated with chymotrypsin at 37°C for 1 hour. SDS-PAGE was carried out by using 12.5% separation gel, the resulting material was transcripted to a PVDF membrane, and N terminus and internal partial amino acid sequences were determined by an amino acid sequencer. The determined amino acid sequences are shown in SEQ.ID.NO: 3 and 4.

[0056]

[Example 3] Cloning of EnHCH gene

Preparation of chromosome DNA from the genus *Enterobacter* DS-S-75 strain (FERM BP-5494) belonging to the genus *Enterobacter* was cultured in 5 ml of a PYG medium for 20 hours, and cells were recovered by centrifugation. The cells were suspended in 490 ml of a TE solution (10 mM Tris-hydrochloride, pH 8.0, 1 mM EDTA), 30 ml 10% SDS and 50 ml of 20 mg/ml protease K were added thereto, and the mixture was reacted at 50°C for 1 hour. Thereafter, extraction was carried out with an equal amount of phenol: chloroform:isoamyl alcohol (25:24:1), 0.1-fold amount of 3 M sodium acetate solution was added thereto, and 0.6-fold amount of 2-propanol was quietly layered. As a result, DNA generated at an interface was recovered by a glass lod by wounding it in a string state.

[0057]

The obtained DNA was dissolved in 500 ml of a TE solution. About 1.1 mg of chromosomal DNA was obtained.

[0058]

(2) Preparation of probe

PCR primer having a DNA sequence expected from amino acid sequences of SEQ.ID.NO: 3 and 4 determined by an amino acid sequencer was synthesized (as shown in SEQ. ID. NO: 5 and 6) by considering codon degeneration, 40 cycles of PCR (thermal denaturation: 96°C for 30 seconds, annealing: 50°C for 1 minute, elongation reaction: 72°C for 1 minute) were carried out by using ExTaq DNA polymerase (available from Takara Shuzo Co., Ltd.) and 50 ng of chromosome DNA extracted in (1) as a template, then, after cooling to 4°C, amplified DNA was confirmed by agarose gel electrophoresis, and a DNA fragment was recovered by agarose gel according to the conventional manner. Then, the DNA fragment was subjected to subcloning to pUC118 (available from Takara Shuzo Co., Ltd.), and the base sequence was determined by a DNA sequencer. As a result, in the determined DNA base sequence, a region which encodes a partial amino acid sequence (SEQ.ID.NO: 3 and 4) previously determined can be determined, so that the obtained amplified DNA was labeled by ^{32}P according to the conventional manner to make a probe DNA.

[0059]

(3) Preparation of restriction enzyme map

The chromosome DNA of the extracted DS-S-75 strain was cut by various kinds of restriction enzymes, after subjecting to electrophoresis by using 1% agarose gel, it was transcripted to a nitrocellulose membrane. After air drying, hybridization reaction was carried out by using the probe prepared in (2) at 65°C for 20 hours.

[0060]

The membrane subjected to hybridization was washed with (1) 2 x SSC, 65°C for 15 minutes and (2) 1 x SSC, 65°C for 30 minutes, and autoradiogram was taken by attaching thereto an X-ray film and a sensitizing paper. As a result, by comparing various signals to each other, a restriction enzyme map at a neighbour of EnHCH gene region was prepared.

[0061]

(4) Cloning of gene from genome library

Genome library in which many kinds of DNA fragments derived from chromosome DNAs of about 6000 base pairs of DS-S-75 strains were inserted into EcoRI portion of plasmid pBluescriptII KS (available from TOYOBO CO., LTD.) was prepared with consideration that whole length of the objective EnHCH gene are contained from the restriction enzyme map prepared in (3). 160 kinds of transformants obtained by introducing the above into *E. coli* DH5α strain were replicated to a nitrocellulose membrane, the membrane was immerced into 0.5 N NaOH to effect lysis of bacteria, and the membrane was neutralized by immercing in 1 M Tris-hydrochloride (pH 7.5). After air drying, hybridization reaction was carried out by using the probe prepared in (2) at 65°C for 20 hours.

[0062]

The membrane subjected to hybridization was washed with (1) 2 x SSC, 65°C for 15 minutes, (2) 1 x SSC, 65°C for 30 minutes and (3) 0.5 x SSC, 65°C for 1 hour, and autoradiogram was taken by attaching thereto an X-ray film and a sensitizing paper. As a result, one transformant which provides a positive signal was obtained.

[0063]

(4) Determination of base sequence

A plasmid was extracted from the above positive strain according to the conventional manner to obtain pBl-EnHCH. Various kinds of insertion gene fragments were deleted from the pBl-EnHCH by exonuclease III and mung bean endonuclease, base sequences of the respective samples were determined by the dideoxy method, the obtained sequences are layerd, and base sequences were determined between BamHI-PstI. The determined base sequence and the amino acid sequence estimated from the base sequence are shown in SEQ.ID.NO: 1 of the sequence table. A schematic drawing of pBl-EnHCH is shown in Fig. 1. In Fig. 1, Determination of the base sequence was carried out between BamHI-PstI portions which sandwitches ORF. ORF encodes 1086bp 362 amino acids. Of these, N-terminal side 25 amino acids are signal peptide.

[0064]

Also, partial amino acid sequences of the purified EnHCH shown in SEQ.ID.NO: 3 and 4 existed in amino acid sequence estimated from the base sequence of the insertion DNA fragment derived from the positive strain, and the partial amino acid sequence was completely accored to each other.

[0065]

[Example 4] Preparation of recombinant plasmid

ATG sequence estimated to be an initiation codon of the EnHCH gene was present at 3 portions. Assembly was carried out by the following operation, so that coupling was carried out under control of a tac promoter of the plasmid pKK223-3 and translation was carried out from the above-mentioned respective initiation codon and N terminus of the purified enzyme

(Fig. 2 and Fig. 3). To provide a EcoRI recognizable sequence to just upstream of these respective ATG sequences, PCR primers shown in SEQ.ID.NO: 9 to 12 were designed, and M13 primers were combined, 30 cycles of PCR (thermal denaturation: 96°C for 30 seconds, annealing: 50°C for 30 seconds, elongation reaction: 72°C for 1 minute) was carried out by using 50 ng of pBl-EnHCH1 as a template (N terminus of the purified enzyme was substituted by methionine (Fig. 2)). In Fig. 2, with regard to three kinds of ATG sequences estimated to be initiation codons, a PCR primer in which EcoRI was added to just upstream was designed, and PCR was carried out in combination with M13 primer derived from pUC118. Also, to effect translation from valine which is the N terminus of the purified enzyme, PCR primer in which valine was substituted by methionine was designed, and PCR was carried out in the same manner. After cooling to 4°C, amplified DNA was confirmed by agarose gel electrophoresis, and various kinds of DNA fragments were recovered by agarose gel according to the conventional manner. Then, these DNA fragments were smoothened by using a BKL kit (available from Takara Shuzo Co., Ltd.) and subjected to phosphorylation, and subjected to subcloning at a HincII restriction enzyme portion of pUC118. With regard to the respective samples, about 400 bases' sequences at upstream were determined by a DNA sequencer.

[0066]

The obtained four kinds of plasmids were digested by restriction enzymes Tth111I and PstI, Tth111I-PstI fragment of pBl-EnHCH was inserted according to the conventional manner, and further, EcoRI-PstI digested fragment was inserted into the same portion of the pKK223-3 (available from Pharmacia Co.) vector to obtain expression plasmids pKK-EnHCH1, pKK-EnHCH2, pKK-EnHCH3 and pKK-EnHCH4 (Fig. 3).

[0067]

[Example 5] Preparation of recombinant *E. coli*

Competent cells of *E. coli* JM109 and DH5α were prepared, and transformed by pKK-EnHCH to obtain recombinated *E. coli* JM109 (pKK-EnHCH1) and DH5α (pKK-EnHCH1). Incidentally, no

DH5α (pKK-EnHCH2) could be obtained. As control samples, JM109 (pKK-223-3) and DH5α (pKK223-3) which had been transformed only by a vector were obtained.

[0068]

[Example 6] Expression of EnHCH gene in recombinant *E. coli*

(1) Preparation of culture broth

The transformant obtained in Example 5 was cultured under aerobic conditions in a 5 ml of a LB medium (1% polypeptone, 0.5% yeast extract and 1% sodium chloride) placed in a test tube at 37°C for 20 hours. As control samples, JM109 strain and DH5α strain each transformed by pKK223-3 were cultured in the same manner. Also, with regard to DS-S-75 strain belonging to the genus *Enterobacter*, it was seed cultured under aerobic conditions in a 5 ml of a PYG medium (1% polypeptone, 1% yeast extract and 1% glycerin) placed in a test tube at 30°C for 20 hours, and cultured samples were obtained from the respective strains.

[0069]

(2) Evaluation of hydrolysis activity of recombinant

To 250 mM Tris sulfate buffer was added p-nitrophenyl butyrate, so that the final concentration became 0.05% (v/v), to prepare a reaction solution. To 3 ml of the reaction solution was added 20 ml of the cultured sample prepared as mentioned above, and the mixture was reacted at 30°C and increase in absorbance at 400 nm derived from p-nitrophenol which had been formed by the hydrolysis reaction was measured. The results are shown in Table 2. Incidentally, 1 U represents a formed amount of 1 μmol of p-nitrophenol per 1 minute at 30°C and specific activity per the prepared culture broth was calculated.

[0070]

[Table 2]

Name of strain	Specific activity (U/ml)
DS-S-75 strain belonging to the genus *Enterobacter*	3.16
JM109 (pKK-EnHCH1)	12.6
JM109 (pKK-EnHCH2)	13.8
JM109 (pKK-EnHCH3)	2.76
JM109 (pKK-EnHCH4)	2.37
JM109 (pKK223-3)	0
DH5α (pKK-EnHCH1)	34.0
DH5α (pKK223-3)	0

[0071]

As a result, *E. coli* transformed by the pKK-EnHCH had a hydrolysis activity, and activities of JM109 (pKK-EnHCH1), JM109 (pKK-EnHCH2) and DH5α (pKK-EnHCH1) had markedly improved as compared to that of DS-S-75 strain belonging to the genus *Enterobacter*. In particular, hydrolysis activity of DH5α (pKK-EnHCH1) per a culture broth was about 10.8-fold.

[0072]

Among four strains of JM109 (pKK-EnHCH1), JM109 (pKK-EnHCH2), JM109 (pKK-EnHCH3) and JM109 (pKK-EnHCH4), JM109 (pKK-EnHCH2) showed the highest activity. Also, an SD (Shine Dalgarno) sequence to which ribosome is linked can be found at just upstream side of the second ATG sequence of the base sequence shown in Fig. 2, so that it is suggested that an inherent initiation codon of the EnHCH gene is to be the second ATG (SEQ.ID.NO: 1). That is, a methionine residue at the second portion of the amino acid sequence shown in Fig. 2 is the N terminus of the EnHCH to be inherently translated in the DS-S-75 strain.

[0073]

(3) SDS-PAGE of recombinant protein

Cultured samples of JM109 (pKK-EnHCH1), JM109 (pKK-EnHCH2), JM109 (pKK-EnHCH3) and JM109 (pKK-EnHCH4) prepared in (1) were harvested by centrifugation, and after suspending with 20 mM potassium phosphate buffer, they were pulverized by ultrasonic wave. Each 5 μg of extracted protein samples and EnHCH purified from DS-S-75 strain belonging to *Enterobacter* were applied to SDS-PAGE (10% separation gel), and stained by Coomassie Brilliant Green. As a result, in either of recombinant enzymes of the recombinant *E. coli*, a band of the recombinant EnHCH was confirmed at the same position with that of the EnHCH purified from DS-S-75 strain belonging to *Enterobacter* (Fig. 4). Molecular weight marker to beused and the molecular weight are shown below. Phosphorylase B (97,400), bovine serum albumin (66,200), ovoalbumin (45,000), carbonic anhydrase (31,000) and tripsin inhibitor (21,500).

[0074]

As shown in Example 2, N terminus amino acid of the purified enzyme was valine. Also, at the N terminus side of the estimated amino acid sequence of the EnHCH shown by SEQ.ID.NO: 2, many hydrophobic residues, positive-charged residues and Ala-Xaa-Ala sequence as a recognized sequence to be cut are present. Thus, it is suggested that the amino acid sequence from translation initiating methionine to the 25th residue is to be a signal peptide (SEQ.ID.NO: 7). Also, as a result of SDS-PAGE, it was clarified that cleavage of the signal peptide is also done in *E. coli*. Also, activities of JM109 (pKK-EnHCH1) and JM109 (pKK-EnHCH2) are higher as compared to those of JM109 (pKK-EnHCH3) and JM109 (pKK-EnHCH4), so that the signal peptide can be said to be important for stable expression thereof in *E. coli*.

[0075]

[Example 7] Optical resolution of carboxylic ester by recombinant *E. coli*

(1) Optical resolution of methyl 4-chloro-3-hydroxybutanoate

In 300 ml volume of an Erlenmeyer flask was charged 60 ml of a LB medium containing ampicillin, and DH5α (pKK-EnHCH1) was subjected to shake culture at 37°C for 16 hours. Also, as a control, DH5α (pKK223-3) transformed only by a vector was prepared. Moreover, DS-S-75 strain belonging to the genus *Enterobacter* was cultured in 60 ml of a PYG medium. To the respective culture brothes were added calcium carbonate and racemic methyl 4-chloro-3-hydroxybutanoate as substrates final concentrations of which became 5% (w/v) and 8% (w/v), respectively, and the mixture was reacted at 30°C for 1 hour under shaking. Also, DS-S-75 strain belonging to the genus *Enterobacter* was further reacted for 24 hours under shaking with a substrate a final concentration of which became 2%. After completion of the reaction, the cells were removed by centrifugation, and a concentration of the methyl 4-chloro-3-hydroxybutanoate remained in the reaction mixture was analyzed by gas chromatography (column carrier: PEG20M, 60-80 mesh). Further, the above-mentioned cell-removed solution was extracted with an equal amount of ethyl acetate, and an optical purity of the methyl 4-chloro-3-hydroxybutanoate remained in the mixture was measured by gas chromatography using G-TA (0.25 mm x 30 m) manufactured by ASTEC INC. As a result, a retension time was 14.7 minutes for R isomer and 15.7 minutes for S isomer. Analytical conditions: column temperature: 110°C, detector temperature: 200°C, carrier gas: nitrogen; flow rate: 0.8 ml/min, detector: FID; sprit ratio: 100/1.

[0076]

Moreover, the mixture was extracted twice with ethyl acetate, and the aqueous fraction was concentrated by an evaporator. The concentrate was dehydrated by anhydrous magnesium sulfate to obtain syrup of 3-hydroxy-γ-butyrolactone. To 10 ml of syrup were added 500 ml of 1,2-dichloroethane and 100 ml of trifluoroacetic acid anhydride and the mixture was allowed to stand for 30 minutes to carry out trifluorination. The solvent was removed under reduced pressure, the residue was dissolved in ethanol, and an optical purity of the 3-hydroxy-γ-butyrolactone was measured by gas chromatography using G-TA (0.25 mm x 30 m). A retension time was 18.7 minutes for R isomer and 19.9 minutes for S isomer. Analytical conditions: column temperature: 120°C, detector temperature: 200°C, carrier gas: nitrogen; flow rate: 0.8 ml/min, detector: FID; sprit ratio: 100/1.

[0077]

As a result, a reaction rate of DH5α (pKK-EnHCH1) was improved as compared to that of the gene donor (DS-S-75 strain belonging to the genus *Enterobacter*). Also, it had a high stereoselectivity, and both of R-methyl 4-chloro-3-hydroxybutanoate and formed S-3-hydroxy-γ-butyrolactone had high optical purities, and almost all amount of the S-ethyl 4-chloro-3-hydroxybutanoate had been converted into S-3-hydroxy-γ-butyrolactone. Also, DH5α (pKK223-3) had no optical resolution ability. After the reaction, measured results of an optical purity of the remaining R-methyl 4-chloro-3-hydroxybutanoate, a yield thereof when an amount of the racemic methyl 4-chloro-3-hydroxybutanoate before initiation of the reaction as 100%, and an optical purity of the formed S-3-hydroxy-γ-butyrolactone are shown in Table 3.

[0078]

[Table 3]

	Methyl 4-chloro-3-hydroxybutanoate		3-Hydroxy-γ-butyrolactone
	Optical purity (%ee)	Yield (%)	Optical purity (%ee)
DH5α (pKK-EnHCH1) (1 hr) (substrate 8%)	99.1 (R)	49.2	99.9 (S)
DH5α (pKK-223-3) (1 hr) (substrate 8%)	0	100	Not formed
DS-S-75 (1 hr) (substrate 8%)	11.8 (R)	88.4	N.D.
DS-S-75 (24 hr) (substrate 8%)	99.5 (R)	48.0	95.9 (S)

[0079]

(2) Optical resolution of ethyl 3-hydroxybutanoate

Various kinds of cells were cultured in the same manner as in Example 7 (1), to the respective culture brothes were added calcium carbonate and racemic ethyl 3-hydroxybutanoate as substrates final concentrations of which became 5% (w/v) and 8% (w/v), respectively, and they were reacted at 30°C for 1 hour under shaking. Also, DS-S-75 strain belonging to the genus *Enterobacter* was further reacted for 4 hours under shaking. After completion of the reaction, the cells were removed by centrifugation.

[0080]

A concentration of the ethyl 3-hydroxybutanoate remained in the reaction mixture was analyzed by gas chromatography (column carrier: PEG20M, 60-80 mesh). Further, the above-mentioned cell-removed solution was extracted twice with an equal amount of ethyl acetate, and the solvent of the ethyl acetate layers were removed under reduced pressure to obtain a syrup of ethyl 3-hydroxybutanoate. The obtained product was subjected to trifluorination by trifluoroacetic anhydride, and an optical purity thereof was measured by gas chromatography using G-TA (0.25 mm x 30 m) manufactured by ASTEC INC. A retension time was 8.1 minutes for R isomer and 12.1 minutes for S isomer. Analytical conditions: column temperature: 90°C, detector temperature: 200°C, carrier gas: nitrogen; flow rate: 0.7 ml/min, detector: FID; sprit ratio: 100/1.

[0081]

On the other hand, a concentration of 3-hydroxybutyric acid fractionated in the aqueous layer was also analyzed by gas chromatography (column carrier: PEG20M, 60-80 mesh). At this time, a pH of the aqueous solution was made pH 4 with phosphoric acid. The aqueous layer was concentrated by an evaporator to obtain a syrup of 3-hydroxybutyric acid. In 30 ml volume of an Erlenmeyer flask was charged 50 mg of the syrup, and under ice-cooling, to the syrup were added 122 mg of 4-dimethylaminopyridine, 10 ml of dichloromethane, 100 ml of ethanol and 115 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. After stirring the mixture for 10 minutes, and the mixture was further stirred at room temperature overnight under sealing. The organic layer was washed twice with 1N hydrochloric acid and placed in a dessiccator to remove the solvent to convert the 3-hydroxybutyric acid into ethyl 3-hydroxybutanoate. The resulting product was subjected to trifluorination in the same manner as mentioned above, and an optical purity thereof was measured by gas chromatography.

[0082]

As a result, a reaction rate of DH5α (pKK-EnHCH1) was improved as compared to that of the gene donor (DS-S-75 strain belonging to the genus *Enterobacter*). Both strains had high stereoselectivity. Also, DH5α (pKK223-3) had no optical resolution ability. After the reaction, measured results of an optical purity of the remaining S-ethyl 3-hydroxybutanoate, a yield thereof when an amount of the racemic ethyl 3-hydroxybutanoate before initiation of the reaction as 100%, and an optical purity of the formed R-3-hydroxybutyric acid are shown in Table 4.

[0083]

[Table 4]

	Ethyl 3-hydroxy-butanoate		3-Hydroxy-butyric acid
	Optical purity (%ee)	Yield (%)	Optical purity (%ee)
DH5α (pKK-EnHCH1) (1 hr) (substrate 8%)	99.2 (S)	49.2	99.9 (R)
DH5α (pKK-223-3) (1 hr) (substrate 8%)	0	100	Not formed
DS-S-75 (1 hr) (substrate 8%)	37.1 (R)	72.0	N.D.
DS-S-75 (4 hr) (substrate 8%)	99.1 (R)	49.0	98.2 (R)

[0084]

(3) Optical resolution of ethyl 2-hydroxybutanoate by recombinant *E. coli*

The reaction was carried out in the same manner as in Example 7 (2) except for changing racemic ethyl 3-hydroxy-butanoate with racemic ethyl 2-hydroxybutanoate. Provided that the reaction time was made 24 hours for the recombinant *E. coli*, and 24 and 36 hours for the DS-S-75 strain. After completion of the reaction, in the same manner as in Example 7 (2), the concentration and the optical purity were analyzed. A retension time was 7.4 minutes for R isomer and 7.8 minutes for S-isomer. Optical purity analyses conditions: column temperature: 90°C, detector temperature: 200°C, carrier gas: nitrogen; flow rate, 0.7 ml/min, detector: FID; sprit ratio: 100/1.

[0085]

As a result, a reaction rate of DH5α (pKK-EnHCH1) was improved as compared to that of the gene donor (DS-S-75 strain belonging to the genus *Enterobacter*), and it had high stereoselectivity. After the reaction, measured results of an optical purity of the remaining R-ethyl 2-hydroxybutanoate, a yield thereof when an amount of the racemic ethyl 2-hydroxybutanoate before initiation of the reaction as 100%, and an optical purity of the formed S-2-hydroxybutyric acid are shown in Table 5.

[0086]

[Table 5]

	Ethyl 2-hydroxybutanoate		2-Hydroxybutyric acid
	Optical purity (%ee)	Yield (%)	Optical purity (%ee)
DH5α (pKK-EnHCH1) (24 hr) (substrate 8%)	98.3 (R)	49.0	98.9 (S)
DH5α (pKK-223-3) (24 hr) (substrate 8%)	0	100	Not formed
DS-S-75 (24 hr) (substrate 8%)	77.3 (R)	60.1	N.D.
DS-S-75 (36 hr) (substrate 8%)	99.2 (R)	49.3	95.1 (S)

[0087]

(4) Optical resolution of methyl tetrahydrofuran-2-carboxylate by recombinant *E. coli*

Various kinds of cells were cultured in the same manner as in Example 7 (1), to the respective culture brothes were added calcium carbonate and racemic methyl tetrahydrofuran-2-carboxylate as substrates final concentrations of which became 5% (w/v) and 1% (w/v), respectively, and they were reacted at 30°C for 2 hours under shaking. Also, DS-S-75 strain belonging to the genus *Enterobacter* was reacted in the same manner for 4 hours and 24 hours under shaking. After completion of the reaction, the cells were removed by centrifugation.

[0088]

A concentration of methyl tetrahydrofuran-2-carboxylate remained in the reaction mixture was analyzed by gas chromatography (column carrier: PEG20M, 60-80 mesh). Further, an optical purity thereof was measured by gas chromatography using G-TA (0.25 mm x 30 m) manufactured by ASTEC INC. A retension time was 8.4 minutes for R isomer and 9.5 minutes for S-isomer. Analytical conditions: column temperature: 110°C, detector temperature: 240°C, carrier gas: nitrogen; flow rate, 0.7ml/min, detector: FID; sprit ratio: 100/1.

[0089]

As a result, a reaction rate of DH5α (pKK-EnHCH1) was improved as compared to that of the gene donor (DS-S-75 strain belonging to the genus *Enterobacter*). After the reaction, measured results of an optical purity of the remaining R-methyl tetrahydrofuran-2-carboxylate, and a yield thereof when an amount of the racemic methyl tetrahydrofuran-2-carboxylate before initiation of the reaction as 100% are shown in Table 6.

[0090]

[Table 6]

	Methyl tetrahydrofuran-2-carboxylate	
	Optical purity (%ee)	Yield (%)
DH5α (pKK-EnHCH1) (2 hr) (substrate 1%)	98.1 (R)	39.6
DH5α (pKK-223-3) (2 hr) (substrate 1%)	0	95.0
DS-S-75 (24 hr) (substrate 1%)	95.7 (R)	54.4
DS-S-75 (48 hr) (substrate 1%)	99.9 (R)	46.8

[0091]

By using DH5α (pKK-EnHCH1), R-methyl 4-chloro-3-hydroxy-butanoate, S-3-hydroxy-γ-butyrolactone, S-ethyl 3-hydroxybutanoate, R-3-hydroxybutyric acid, R-ethyl 2-hydroxybutanoate, S-2-hydroxybutyric acid, R-methyl tetrahydrofuran-2-carboxylate, R-ethyl lactate and R-ethyl 4-phenyl-2-hydroxybutanoate can be produced. And yet, they showed stereoselectivity substantially equal or more than that of the gene donor (DS-S-75 strain belonging to the genus *Enterobacter*), and the reaction rate was extremely rapid. That is, by incorporating a gene which encodes EnHCH, a recombinant *E. coli* having high stereoselective hydrolysis activity can be obtained.

[0092]

[Sequence Listing]

SEQUENCE LISTING

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<120> Hydroxy carboxylic ester hydrolase

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Ser Ala Gln Val Thr Arg Asp Thr Leu Gly Thr Met Glu Lys Gln Tyr
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caa cag atg tgg gag aaa gaa aat ggc ccg ctg acg ttg tcg cct ccg      915
Gln Gln Met Trp Glu Lys Glu Asn Gly Pro Leu Thr Leu Ser Pro Pro
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gat gtc gcg gtg ccg gga cga aaa att ccg gta cgg atc tac aac ccg 1107
Asp Val Ala Val Pro Gly Arg Lys Ile Pro Val Arg Ile Tyr Asn Pro
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cat ccg gat ata gca acc ggg gtg att ttc ttc att cat ggt ggg ggg 1155
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Ser Gly Gly Leu Asp Pro Leu Arg Asp Glu Asp Phe Ala Phe Val Ala
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cga ctg aaa agc gcc gga gtg ccg gtc agg cat atc cac ttc ccg ggg      1731
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        35                  40                  45

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Leu Ser Ser Leu Pro Lys Asn Ser Asn Asn Pro Glu Tyr Asn Thr Leu
65                  70                  75                  80

Asp Ser Arg Asp Ala Leu Thr Ala Leu Thr Gln Lys Tyr Val Thr Asp
                85                  90                  95

Lys Gln Ser Ile Ala Arg Ile Ile Asn Val Asp Val Ala Val Pro Gly
            100                 105                 110

Arg Lys Ile Pro Val Arg Ile Tyr Asn Pro His Pro Asp Ile Ala Thr
        115                 120                 125

Gly Val Ile Phe Phe Ile His Gly Gly Gly His Leu Ser Gly Ser Val
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Asp Val Tyr Asp Pro Ile Ala Arg His Leu Ala Ala Ala Thr Gly Asn
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Thr Val Val Ala Val Asp Tyr Arg Arg Ala Pro Glu Ser Pro Tyr Pro
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Glu Gly Leu His Asp Ala Arg Asp Val Leu Met Gln Val Tyr Ala Val
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Leu Asp Gln Asn His Val Pro Trp Lys Pro Gln Leu Thr Leu Ala Gly
        195                 200                 205

Asp Ser Gly Gly Gly Ala Phe Ser Ala Thr Leu Ala Gly Asp Leu Gln
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Thr Glu His Pro Gly Phe Ile Ser Arg Leu Glu Leu Ile Tyr Pro Ser
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[Brief description of the drawings]

[Fig. 1]

A schematic drawing showing EnHCH gene (pBl-EnHCH) which has been subjected to cloning to pBluescriptIIKS obtained by colony hybridization. Determination of the base sequence was carried out between BamHI-PstI portions which sandwitches ORF. ORF encodes 1086bp 362 amino acids. Of these, N-terminal side 25 amino acids are signal peptide.

[Fig. 2]

An explanation of a PCR primer for construction of pKK-EnHCH 1 to 4. With regard to three kinds of ATG sequences estimated to be initiation codons, a PCR primer in which EcoRI was added to just upstream was designed, and PCR was carried out in combination with M13 primer derived from pUC118. Also, to effect translation from valine which is the N terminus of the purified enzyme, PCR primer in which valine was substituted by methionine was designed, and PCR was carried out in the same manner.

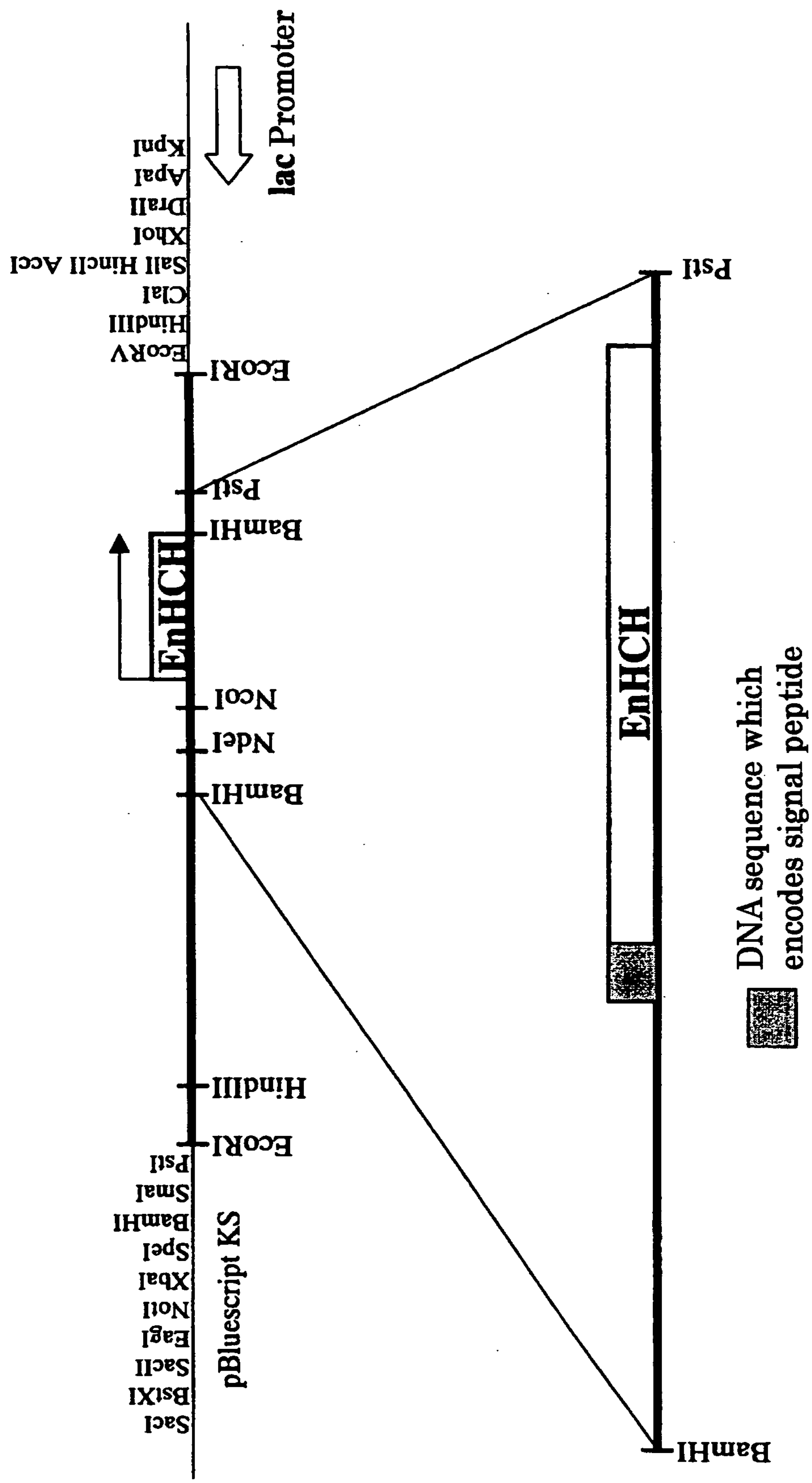
[Fig. 3]

It shows subcloning of a PCR product obtained in Fig. 2 to a plasmid pKK223-3. First, subcloning was firstly carried out to pUC118 at smooth terminus, and about 400 bp of base sequence at upstream side were determined. After confirmation that there is no PCR elongation error, sequences between Tth111I-PstI were replaced by those of pB1-EnHCH, and ligated by EcoRI-PstI to downstream of a tac promoter of pKK223-3.

[Fig. 4]

It is a SDS-PAGE photograph of a recombinant protein. From the lane at the left hand, they are the purified EnHCH derived from the gene donor (DS-S-75 strain), an extract of JM109 (pKK223-3), an extract of JM109 (pKK-EnHCH1), an extract of JM109 (pKK-EnHCH2), an extract of JM109 (pKK-EnHCH3), an extract of JM109 (pKK-EnHCH4), molecular weight markers. A band of the recombinant EnHCH could be confirmed at the same position to that of the purified EnHCH of the gene donor.

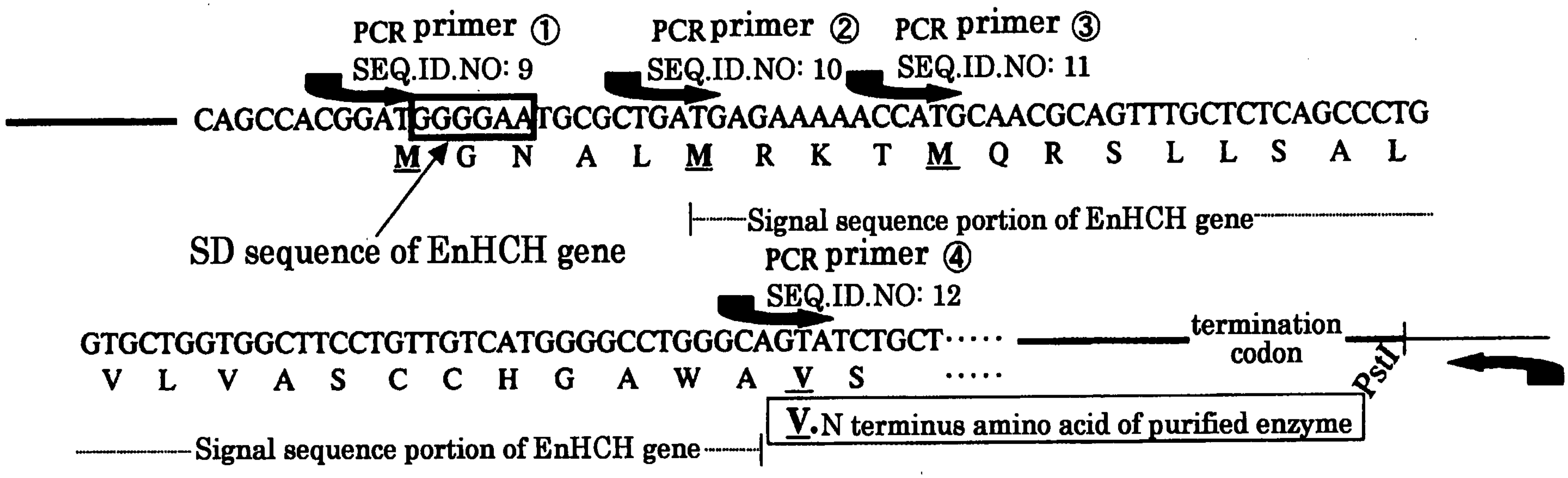
[Fig. 1]



Schematic view of pB1-EnHCH

[Fig. 2]

Construction of pKK-EnHCH1-4 ①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PCR primer ②
SEQ.ID.NO: 10
PCR primer ③
SEQ.ID.NO: 11
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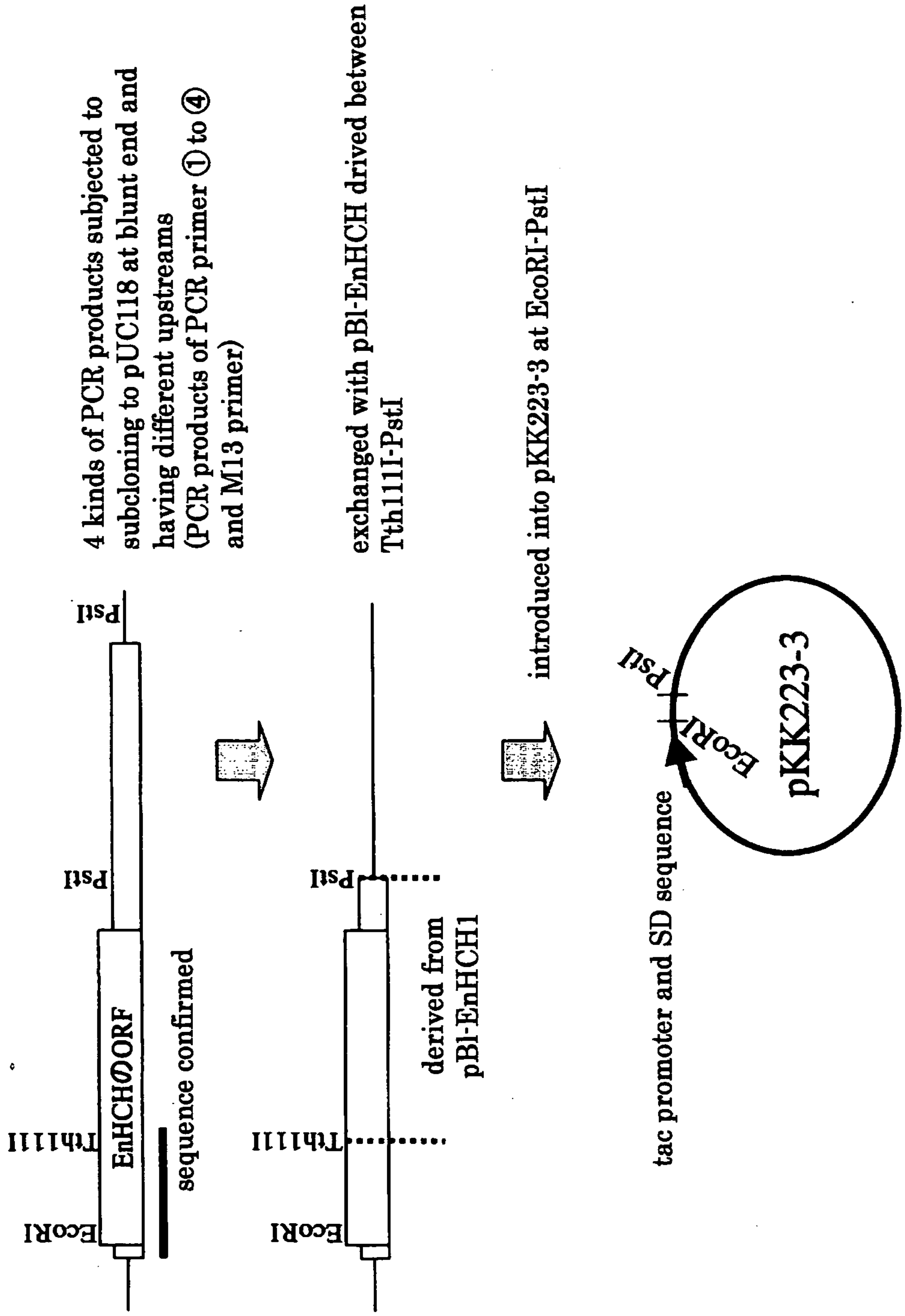
M G N A L M R K T M Q R S L L S A L
SD sequence of EnHCH gene
Signal sequence portion of EnHCH gene
PCR primer ④
SEQ.ID.NO: 12
GTGCTGGTGGCTTCCTGTTGTCATGGGGCCTGGGCAGTATCTGCT·····
V L V A S C C H G A W A V S ·····
termination codon
PstI
V.N terminus amino acid of purified enzyme
Signal sequence portion of EnHCH gene

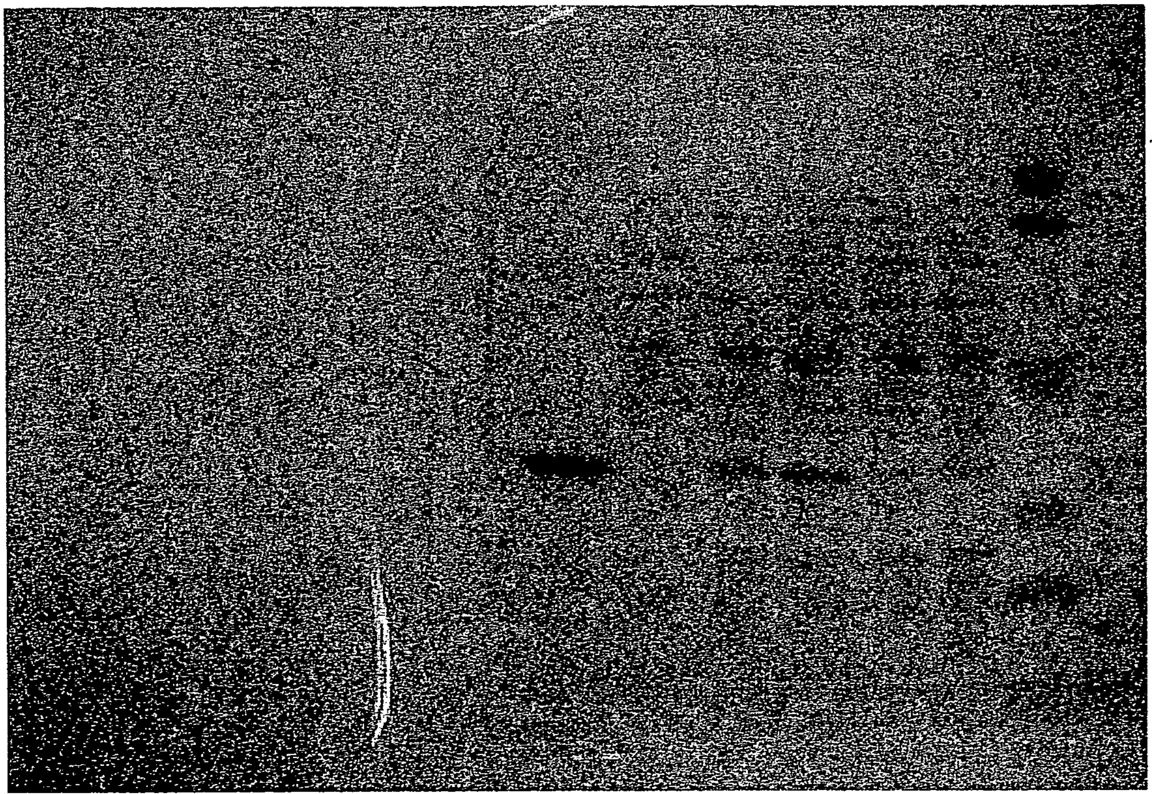
EcoRI portion was provided to respective primers ① to ④
And PCR was carried out in combination with M13 primer.

[Fig. 3]

Construction of pKK-EnHCH1-4 ②


EcoRI
Tth111I
PstI
PstI
EnHCHのORF
sequence confirmed
4 kinds of PCR products subjected to subcloning to pUC118 at blunt end and having different upstreams (PCR products of PCR primer ① to ④ and M13 primer)
EcoRI
Tth111I
PstI
derived from pBI-EnHCH1
exchanged with pBI-EnHCH drived between Tth111I-PstI
introduced into pKK223-3 at EcoRI-PstI
PstI
EcoRI
pKK223-3
tac promoter and SD sequence

[Fig. 4]



[Document name] Abstract

[Summary]

[Problem] To provide a gene having asymmetric hydrolase activity which is useful for synthesis of an optically active carboxylic acid, its antipode ester, and lactone.

[Solving means] A hydroxycarboxylic ester asymmetric hydrolase enzyme (EnHCH) derived from *Enterobacter sp.* DS-S-75 strain (FERM BP-5494) which is bacteria belonging to the genus *Enterobacter*, a EnHCH gene shown by base sequence of SEQ.ID.NO: 1, a gene encoding a protein having an amino acid sequence of SEQ.ID.NO: 2, and *E. coli* DH5α (pKK-EnHCH) deposited to International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology as a deposition No. FERM BP-08466.

[Selective figure] None